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Abbreviations: -

CASA: Computer-assisted semen analysis -

CV: Coefficient of variation

ELISA: Enzyme-linked immunosorbent assay

FAI: Free androgen index

FSH: Follicle stimulating hormone

IQR: Inter quartile range

LH: Luteinizing hormone

PFAA: Perfluorinated alkyl acid

PFOA: Perfluorooctanoic acid

PFOS: Perfluorooctane sulfonic acid

SHBG: Sex hormone binding globulin

Abstract

Background: Perfluorinated alkyl acids (PFAAs) are persistent chemicals with unique water-, dirt-, and oil-repellent properties, and suspected endocrine disrupting activity. The PFAA compounds perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) are found globally in humans, and since they readily cross the placental barrier, *in utero* exposure may be a cause of concern.

Objectives: To investigate whether *in utero* exposure to PFOA and PFOS affects semen quality, testicular volume, and reproductive hormone profile.

Methods: We recruited 169 male offspring (19-21 years old) of a pregnancy cohort established in Aarhus, Denmark in 1988-89, corresponding to 37.6% of the eligible sons. Each provided a semen sample that was analysed for sperm concentration, total count, motility, and morphology, and a blood sample that was used to measure reproductive hormones. As a proxy of *in utero* exposure, PFOA and PFOS were measured in maternal blood samples from pregnancy week 30.

Results: Multivariable linear regression analysis suggested that *in utero* exposure to PFOA was associated with lower adjusted sperm concentration (p trend=0.01) and total sperm count (p trend=0.001), and with higher adjusted levels of luteinizing hormone (LH) (p trend=0.03) and follicle stimulating hormone (FSH) (p trend=0.01). PFOS did not appear to be associated with any of the outcomes assessed, before or after adjustment.

Conclusions: The results suggest that *in utero* exposure to PFOA may affect adult human male semen quality and reproductive hormone levels.

Introduction

Perfluorinated alkyl acids (PFAAs) are a class of chemicals with unique water-, dirt-, and oil-repellent properties, high stability, and resistance to degradation. They are used as surfactants in many industrial processes and consumer products, for example, as oil and water repellents for fabrics and food packaging materials (Kissa 2001). Several PFAAs accumulate in food chains and have been detected in human serum worldwide (Giesy and Kannan 2001; Kannan et al. 2004). Although sources of human exposure are not fully understood, dietary intake is thought to be a major pathway of exposure in general populations, originating either from environmental contamination or migration from food packaging (Tittlemier et al. 2006; Tittlemier et al. 2007). Furthermore, human exposure through drinking water in contaminated areas and dust in indoor environments may be significant (Emmett et al. 2006; Shoeib et al. 2011; Haug et al. 2011).

Two of the most abundant PFAAs in human serum samples are PFOA and PFOS. Their half lives in human blood serum have been estimated to be 3.8 years and 5.4 years, respectively (Olsen et al. 2007). Studies on adult male rats show that PFOA exposure may cause reduced testosterone levels and increased estradiol levels (Lau et al. 2007), and a study on sexually mature mice has indicated that PFOS exposure might affect testicular signalling causing reduced serum testosterone and epididymal sperm counts (Wan et al. 2011). Two cross-sectional studies have reported negative associations of PFOS, or high PFOA and PFOS (combined), with the proportion of morphologically normal spermatozoa in adult men (Joensen et al. 2009; Toft et al. 2012). Furthermore, a study of men attending an *in vitro* fertilization clinic reported that luteinizing hormone (LH) and free testosterone were significantly positively correlated with plasma PFOA, though PFOA was not associated with semen characteristics (Raymer et al. 2011).

The widespread environmental occurrence of PFOA and PFOS, and their ability to cross the placental barrier (Inoue et al. 2004; Fei et al. 2007; Gutzkow et al. 2011), means that exposure of the developing human fetus is inevitable. This is of concern because fetal development of the male reproductive organs may be disturbed by exposure to exogenous factors (Jensen et al. 2010; Palmer et al. 2009). Additionally, rat studies have suggested the existence of a male programming window, corresponding to gestational weeks 8-14 in humans, during which xenobiotic exposure may affect reproductive hormone balance and impact normal male reproductive development (Welsh et al. 2008). To our knowledge, potential associations between prenatal exposure to PFOA and PFOS and adverse effects on the human male reproductive system have not been investigated, though potential effects on immune system development were studied by Grandjean et al. (2012), who reported decreased antibody responses to childhood diphtheria vaccinations in association with prenatal PFOA and PFOS exposures.

The prospective design of the present study enables us to investigate the hypothesis that *in utero* exposure to PFOA and PFOS is associated with reduced semen quality and testicular size, and altered reproductive hormones, in adult men.

Methods

Population

Physical examinations were performed on sons of a pregnancy cohort recruited to a study in 1988-1989 in Aarhus, Denmark. Mothers answered a questionnaire on dietary- and lifestyle habits and provided a blood sample, which was stored in a bio-bank at -20ºC. Of all invited pregnant women, 80% participated (n=965) (Olsen et al. 1995a; Olsen et al. 1995b). In 2008, sons of the pregnancy cohort were invited to answer an internet-based questionnaire on health

and lifestyle habits, and in 2008-2009 they were invited to a physical examination where they donated a semen sample (which they had produced at home), blood samples for reproductive hormone analyses, and self-measured their testicular volumes (Vested et al. 2011). One hundred and seventy-six sons attended, which corresponds to a participation rate of 37.6% of the eligible cohort of sons contacted about the questionnaire in 2008 (n=468). The men provided written informed consent prior to participation, and the study was approved by the regional ethics committee (registration number M-20070157).

Maternal PFOA and PFOS serum concentration measurements were missing for six participants and one was excluded from all statistical analysis due to azoospermia. Hence, the study population included 169 men (Figure 1).

Semen collection and physical examination.

Physical examinations were conducted from February 2008 to September 2009. Semen samples were stored in a heating chamber (37ºC) until semen analysis. All participants provided information on time and date of the semen sample collection and spillage during semen sample collection. Testicular volume self-measurements were performed using a Prader Orchidometer, which, according to a study by Ramlau-Hansen et al. (2007), appears to be a valid method for testicular size measurement (Ramlau-Hansen et al. 2007). The examiner measured the height and weight of each participant. Blood samples were taken between 7.30 $_{AM}$ and 1.30 $_{PM}$.

Semen analysis

Semen analysis was initiated within one hour of ejaculation for 86% of the samples, and all were analysed within two hours. Semen volume was estimated based on weight (1g=1mL). Conventional analysis of motility and sperm concentration was performed by two laboratory

technicians (one in the beginning and the other at the end of the study) according to World Health Organization guidelines from 1999 (World Health Organization 1999). Motility, sperm concentration, and total sperm counts also were assessed by computer-assisted semen analysis (CASA), using CRISMAS Clinical software version 4.6 (Image House Medical, IHMedical A/S, Copenhagen, Denmark), as previously described (Vested et al. 2011). The laboratory continuously participated in the European Society for Human Reproduction and Embryology external quality control program. All tests were in agreement with the quality control standards. Sperm morphology was classified as normal or abnormal using "Strict criteria" (Menkveld et al. 1990).

Blood sample analysis

Maternal serum samples were collected in pregnancy week 30 in 1988-1989 and stored at -20ºC until analysis in 2010-2011. Concentrations of PFOA and PFOS were determined by column-switching, isotope dilution LC-MS/MS methodology at the Division of Environmental Medicine, Norwegian Institute of Public Health in Oslo, Norway, as previously described (Haug et al. 2009). Limits of quantification were 0.05ng/mL. Quality of the analytical procedure was monitored by analysing in-house quality control samples (n=18) as well as human serum samples from an inter-laboratory comparison exercise (n=3). Coefficients of variation (CV) for PFOA and PFOS for the in-house quality control samples were 11% and 4.4%, respectively, and inter-laboratory comparison results were within one standard deviation of the consensus values. Serum concentrations of sex hormone-binding globulin (SHBG) were measured using a solid-phase two-site chemiluminescent immunometric assay (IMMULITE ® 2000, Siemens Healthcare Diagnostics Products Ltd., Gwynedd, UK) with CV of 4.5-4.7%, and concentrations of LH, follicle stimulating hormone (FSH), estradiol, and testosterone were analysed using

immunoassays (cobas® 6000 e601, Roche Diagnostics, Mannheim, Germany) at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark, with CVs of 1.1-2.4%, 1.9-2.1%, 1.5-2.9%, and 2.2-4.5%, respectively. Undiluted inhibin-B was measured using a commercially available ELISA (Oxford Bio-innovation Ltd, Oxfordshire, England, UK) with a detection of 20 pg/ml and CV <7%, at the Laboratory of Reproductive Biology, Juliane Marie Centre for Women, Children and Reproduction, University Hospital of Copenhagen, Denmark. Measurements below the detection limit for LH (n=1), FSH (n=1), and estradiol (n=4) were recoded to half the detection limit (0.15 IU/L, 0.15 IU/L, and 0.025 nmol/L, respectively).

Statistical analysis

Outcome variables included semen parameters [sperm concentration, total sperm count, semen volume, percentage progressive spermatozoa (rapidly progressive + slowly progressive), and percentage morphologically normal spermatozoa], mean testicular volume (estimates were comparable for left and right volumes separately), and reproductive hormones (testosterone, estradiol, LH, FSH, SHBG, and inhibin B). Free androgen index (FAI) was calculated as (testosterone/ SHBG) × 100.

Differences across tertiles of maternal PFOA and PFOS exposure were estimated using one-way ANOVA tests and Chi^2 tests. Crude trends were tested by Spearman's rank correlation test and adjusted trends by multivariable regression analyses by entering PFOA and PFOS as continuous variables in the model. Outcome variables were natural logarithm (ln) transformed before multivariable regression analysis, and participants were divided into three groups according to tertiles of maternal PFOA and PFOS concentrations, or according to quintiles (for associations with a significant linear trend). Differences between the two upper tertiles versus the lowest tertile were tested by Two-sample Wilcoxon rank-sum (Mann-Whitney) test (crude

results) and by multivariable regression analysis for each of the outcome variables with low PFOA and PFOS groups as referents, and results are presented as adjusted percentage differences with 95% confidence intervals (95% CI), which were calculated based on the log scale output from the regression analysis. Multivariable regression results were adjusted for the following *a priori* selected potential confounders: history of reproductive tract disease [cryptorchidism, hypospadias, inguinal hernia, varicocele, testicular hydrocele, incarcerated hernia, phimosis, torsio testis, chlamydia, gonorrhea, and epididymitis, combined into one dichotomous variable (any versus none)], body mass index (BMI, continuously in kg/m^2), smoking status (current and party smoker/ ex and never smoker), smoking by the participant's mother during pregnancy (yes/ no), and socioeconomic status at birth (total annual income for the household in 1987 <200,000 DKR or ≥200,000 DKR). Additionally, sperm concentration, total sperm count, percentage progressive spermatozoa, semen volume, and testicular volume were adjusted for abstinence time (≤48 hours, 49-120 hours, ≥121 hours); sperm concentration for spillage (yes or no); percentage progressive spermatozoa for time from ejaculation to semen analysis (continuous, in minutes); and reproductive hormones for time of day of blood sampling (7:30-9:29 $_{AM}$, 9:30-11:29 $_{AM}$, ≥11:30 $_{AM}$). Participants reporting spillage during semen sample collection (n=45) were excluded from analyses of total sperm count and semen volume. All statistical analyses were performed using Stata 11.2 software (Stata Corporation, College Station, TX, USA), and a two-tailed probability level of $P<0.05$ was considered statistically significant.

Results

The 169 study participants had a median age of 20 (range 19-21) years. The mothers' median (p25–p75) plasma concentrations of PFOA and PFOS were 3.8 ng/mL (2.8–4.7) and 21.2

ng/mL (17.4–26.5), respectively, and PFOA and PFOS concentrations were highly correlated (Spearman's rho=0.73, p<0.0001).

Characteristics of the study participants according to tertiles of *in utero* PFOA and PFOS exposure are shown in Table 1. Characteristics did not differ substantially, except that participants in the high PFOA tertile were less likely to have mothers who smoked during pregnancy than those in the medium and low PFOA tertile groups.

Trend tests on crude sperm concentration and total count did not indicate significant associations with PFOA exposure (Table 2). However, estimates from multivariable regression models indicated significant negative trends for sperm concentration and total sperm count in association with *in utero* exposure to PFOA, and a 34% reduction (95% CI: -58, 5%) in sperm concentration and 34% reduction (95% CI: -62, 12%) in total count estimated for the highest exposure group compared with the lowest (Table 2). A sub-analysis of associations with quintiles of PFOA exposure suggested that the statistically significant negative linear trends for sperm concentration and total sperm count were largely driven by pronounced decreases of sperm concentration and total sperm count among men in the 5th quintiles (see Supplemental Material, Table S1). Computer assisted semen analysis (CASA) using CRISMAS Clinical 4.6 software (Image House, Copenhagen, Denmark) supported the results from the manual assessment of semen analysis (Supplemental Material, Table S2). For comparison, men in the high PFOA tertile had 33% (95% CI: -54, -1%) lower CASA sperm concentration and 34% (95% CI: -58, 6%) lower CASA total sperm counts compared to men in the low PFOA group.

No significant trends of associations between percentage progressive spermatozoa and PFOA were indicated in either crude or adjusted results based on the manual assessment (Table 2). However, in the comparison between tertiles of exposure, crude results showed that sons in

the medium PFOA group had 10% lower percentage progressive spermatozoa than sons exposed to the lowest PFOA levels (p=0.02). After adjustment, this association was no longer statistically significant (p=0.06). The CASA semen analysis provided somewhat stronger indications of an association between PFOA exposure and percentage progressive spermatozoa with a significant trend in the adjusted analyses and a 13% relative decrease (95% CI: -23, -2%) in the percentage of progressive motile spermatozoa in the high PFOA group compared to the low PFOA group (see Supplemental Material, Table S2).

There were no significant trends or differences between exposure groups estimated for *in utero* exposure to PFOA in association with sperm morphology, semen volume, or testicular volume (Table 2).

For the reproductive hormones, there was a trend of higher crude levels of FSH with higher *in utero* exposure to PFOA, which remained statistically significant after transformation and adjustment (Table 2). Based on the crude analysis of differences between tertiles, sons exposed to the highest tertile of PFOA had 27% higher FSH levels (p=0.03), compared to sons exposed to the lowest tertile of PFOA (Table 2). After adjustment, FSH levels were estimated to be 31% higher (95% CI: 5, 64%) in the high PFOA group compared with the reference group. LH was not statistically significantly associated with PFOA exposure based on the crude trend test, but adjusted analyses showed a trend of higher LH with higher *in utero* exposure to PFOA, and a 24% (95% CI: 4, 48%) higher LH level estimated for the highest versus lowest PFOA exposure group.

There were no statistically significant associations between *in utero* exposure to PFOA and any of the other reproductive hormones or FAI (Table 2). Analysis of *in utero* exposure to PFOS showed no significant trends or associations for any of the measured semen outcomes or

reproductive hormones in either crude or adjusted analyses (Table 3, Supplemental Material, Table S3). A sub-analysis adjusting PFOA trend analyses for PFOS did not alter the observed associations significantly (data not shown).

Discussion

To the best of our knowledge, this is the first longitudinal study to indicate associations between *in utero* exposure to PFOA and semen quality and reproductive hormone levels in adult men. Trends of lower sperm concentration and total sperm count and higher FSH and LH with higher *in utero* exposure to PFOA were observed. Estimates did not support associations between *in utero* exposure to PFOS and any of the investigated parameters.

For sperm concentration, total count, and LH there were no statistically significant associations with PFOA in nonparametric models based on the crude, untransformed outcome data, but significant associations were estimated after transforming the outcome data to obtain a normal distribution of residuals and adjusting for potential confounders. Adjustment of the transformed data for potential confounders did not alter the strength of the associations (see Supplemental Material, Table S4), indicating that it was the transformation required to perform parametric linear regression analysis rather than adjustment that caused findings to differ between crude and transformed and adjusted data.

The longitudinal design is a major strength, enabling us to estimate effects of PFOA and PFOS exposure during the crucial prenatal period of male reproductive organ programming and development on markers of adult reproductive capacity. Considering that correlations between maternal serum levels of PFOA and PFOS during pregnancy and offspring cord blood levels are generally good (r>0.82 for PFOA and r>0.72 for PFOS) (Fei et al. 2007; Gutzkow et al. 2011),

and that PFAA measurements from different trimesters have been shown to be highly correlated (r=0.88 for PFOA and r=0.87 for PFOS) (Fei et al. 2007), we believe that the measured exposure in pregnancy week 30 is a good proxy for *in utero* exposure.

Selection bias is caused by differences in characteristics between participants and non participants of a study. It is a potential concern due to the low participation rate (37.6%) and because former studies have shown that men with fertility problems are more likely to participate in reproduction studies (Bonde et al. 1996). However, in this study participants had no knowledge about their mothers PFAA exposure levels during pregnancy and median (inter quartile range (IQR)) maternal levels of PFOA were almost identical for sons not participating in follow-up [3.7 ng/mL (1.8)], those who completed questionnaires [3.8 ng/mL (2.1)], and physical examination participants [3.8 ng/mL (1.9)]. Additionally, due to the participants' young age and lack of reproductive experience, it is unlikely that the majority of participants had any knowledge about whether they had fertility problems or not and hence, it is unlikely that participation is related to fecundity.

Two human cross-sectional studies reported that exposure to PFOA and PFOS was negatively associated with percentage morphologically normal spermatozoa (Joensen et al. 2009; Toft et al. 2012), whereas we did not observe associations between *in utero* exposure to PFOA or PFOS and percentage morphologically normal spermatozoa in our study population. One possibility to explain these differences is that relevant time windows of exposure for effects on morphology and sperm production may differ, such that the former is influenced more by current exposures while the latter may be primarily influenced by developmental exposures. This is plausible, since the capacity for sperm production later in life is determined during sexual organ development in fetal life whereas the morphology and motility of spermatozoa are determined

during sperm production in adolescence and adulthood. Additionally, the association with sperm concentration and total sperm count suggests an effect of PFOA on Sertoli cell development, since proliferation of Sertoli cells *in utero* is a determinant for spermatozoa development later in life (Sharpe et al. 2003), but the exact mechanism remains unclear. Furthermore, we observed a positive trend between *in utero* exposure to PFOA and LH and FSH levels in adult men, which supports the general notion that high levels of gonadotrophins are expected to be associated with low sperm concentration and total sperm count and corresponds with that men in the high PFOA tertile had a tendency towards lower sperm concentration and total sperm count compared to men in the low tertile (de Kretser 1979; de Kretser et al. 1989; Appasamy et al. 2007; Gordetsky et al. 2011; Tuttelmann et al. 2009).

We estimated negative associations between the proportion of motile spermatozoa and PFOA exposure, particularly when the outcome was measured using computer assisted semen analysis (CASA). CASA semen concentration and motility results differed substantially from the conventional semen analysis results, as described in Vested et al. 2011, which may explain the apparent differences in the multivariable regression results between conventional semen analysis and CASA.

Studies on rats exposed *in utero* to ammonium PFOA have not suggested adverse effects on the male reproductive system (Butenhoff et al. 2004; York et al. 2010) and only few human studies have so far investigated possible effects of *in utero* exposure to xenobiotic compounds on male reproduction. A study investigating early-life exposure to low doses of dioxin reported that associations with reproductive outcomes in adult males appeared to be related to postnatal exposure via breastfeeding, rather than exposure *in utero* (Mocarelli et al. 2011). Like dioxins, PFAAs are also transferred from mother to child by breastfeeding, which would result in

postnatal PFAA exposure that would be correlated with prenatal exposure (Haug et al. 2011). Therefore, we cannot exclude the possibility that the observed associations may be at least partially explained by effects of postnatal exposure rather than prenatal exposure.

Background levels of PFOA and PFOS have been declining in the Western world, especially during the last decade where percentage decline in geometric mean concentrations from 2000-2001 to 2010 have been reported to be 76% for PFOS and 48% for PFOA (Olsen et al. 2012). However, the levels measured in 1988-1989 in the mothers of the present study participants (median levels of 3.8 ng/mL and 21.2 ng/mL for PFOA and PFOS, respectively) were lower than median levels in samples collected during 2003-2004 in Denmark (4.9ng/mL and 24.5ng/mL, respectively) (Joensen et al. 2009) and the USA (9.2ng/mL and 32.3ng/mL, respectively) (Raymer et al. 2011). Hence, *in utero* exposures in our study population were similar to or slightly lower than levels experienced by children born 15 years later.

Our findings suggest that the fetal male reproductive system may be sensitive to background exposure levels of PFOA. Corroboration by other studies would further support the hypothesis that PFOA may be a reproductive toxicant that may be contributing to reduced semen quality in adult men.

Conclusions

In summary, although crude analyses only suggested a trend of higher FSH with increasing PFOA exposure, multivariate analyses suggest a trend of lower sperm concentration and total count and a trend of higher LH and FSH with higher *in utero* exposure to PFOA. Prenatal exposure to PFOS was not related to any of the semen parameters, testicular volume, or reproductive hormones.

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Table 1: Characteristics of 169 participants and biologic samples according to PFOA and PFOS concentrations in maternal serum at gestational week 30.

Characteristic	Low PFOA (1.26-3.15 ng/mL) n=57	Medium PFOA (≥3.15-4.40 ng/mL) n=56	High PFOA (≥4.40-16.57 ng/mL) n=56	p-value	Low PFOS (7.47-18.78 ng/mL) n=57	Medium PFOS (≥18.78-24.31 ng/mL) n=56	High PFOS (≥24.31-54.28ng/mL) n=56	p-value
Person related characteristics of sons								
Body mass index (kg/m2), mean ± SD	22.7 ± 2.6	23.1 ± 3.1	22.5 ± 3.0	0.6[b]	22.8 ± 2.4	22.6 ± 2.9	23.0 ± 3.4	0.7[b]
History of reproductive tract disease[a]	6 (10.5)	11 (19.6)	8 (14.3)	0.7[c]	5 (8.8)	9 (16.1)	11 (19.6)	0.7[c]
Current/ occasional smoker	26 (45.6)	28 (50.0)	28 (50.0)	0.8[c]	29 (50.9)	30 (53.6)	23 (41.1)	0.3[c]
Person-related characteristics of mothers								
Mother smoking during pregnancy	20 (35.1)	22 (39.3)	11 (19.6)	0.05[c]	22 (38.6)	18 (32.1)	13 (23.2)	0.2[c]
Socioeconomic status (Total annual household income 1987)								
<200,000 DKR	23 (40.4)	21 (37.5)	16 (28.6)	0.6[c]	20 (35.1)	23 (41.1)	17 (30.4)	0.8[c]
>200,000 DKR	31 (54.4)	29 (51.8)	35 (62.5)	0.6[c]	32 (56.1)	28 (50.0)	35 (62.5)	0.8[c]
Semen and blood-related characteristics								
Duration of abstinence								
≤48 hours	34 (59.7)	27 (48.2)	31 (55.4)	0.6[c]	37 (64.9)	25 (44.6)	30 (53.6)	0.1[c]
49 hours - 5 days	22 (38.6)	25 (44.6)	23 (41.1)	0.6[c]	20 (35.1)	27 (48.2)	23 (41.1)	0.1[c]
> 5 days	1 (1.8)	4 (7.1)	2 (3.6)	0.6[c]	0 (0)	4 (7.1)	3 (5.4)	0.1[c]
Min. from ejaculation to semen analysis, mean ± SD	45.9 ± 16.1	41.9 ± 21.2	41.2 ± 20.5	0.4[b]	42.0 ± 17.0	45.1 ± 19.9	42.2 ± 21.1	0.7[b]
Spillage at semen sampling	16 (28.1)	18 (32.1)	11 (19.6)	0.3[c]	16 (28.1)	14 (25.0)	15 (26.8)	0.9[c]
Time for blood sampling								
7.30-9.29	16 (28.1)	22 (39.3)	16 (28.6)	0.4[c]	18 (31.6)	16 (28.6)	20 (35.7)	0.1[c]
9.30-11.29	28 (49.1)	23 (41.1)	32 (57.1)	0.4[c]	22 (38.6)	32 (57.1)	29 (51.8)	0.1[c]
11.30-	13 (22.8)	8 (14.3)	8 (14.3)	0.4[c]	15 (26.3)	7 (12.5)	7 (12.5)	0.1[c]

[a]History of reproductive tract disease include: cryptorchidism, hypospadias, inguinal hernia, varicocele, testicular hydrocele, incarcerated hernia, phimosis, torsio testis, chlamydia, gonorrhea, and epididymitis combined into one variable (yes/no). [b]One-way ANOVA test of differences across tertiles of maternal PFOA and PFOS exposure, [c] Chi2-test of differences across tertiles of maternal PFOA and PFOS exposure. Note: Values are number (percentage) unless otherwise stated

Table 2: Semen-, testicular size-, and reproductive hormone characteristics for 169 young Danish men stratified by tertiles of maternal pregnancy week 30 serum PFOA concentrations.

Parameter	n	Low PFOA: Median (p25–p75)	Medium PFOA: Median (p25–p75)	High PFOA: Median (p25–p75)	Spearman corr. coeff.[a]	Trend p-value[a]	Medium PFOA: % difference from low (95% CI) [b]	High PFOA: % difference from low (95% CI) [b]	Adjusted β-coeff.[c] (SE)	Adjusted trend p-value[c]
Sperm concentration (mio/mL)	168	33 (23–59)	46 (14–71)	30 (10–66)	-0.11	0.15	-7 (-42, 47)	-34 (-58, 5)	-0.11 (0.04)	0.01
Total sperm count (mio)	123	121 (59–187)	144 (59–204)	74 (31–223)	-0.15	0.10	2 (-42, 81)	-34 (-62, 12)	-0.20 (0.06)	0.001
Semen volume (mL)	123	3 (2– 4)	3 (2–4)	3 (2–4)	0.09	0.34	14 (-8, 41)	12 (-8, 37)	-0.01 (0.02)	0.54
Percentage progressive spermatozoa	167	67 (60–77)	60 (51–70)	66 (52–72)	-0.14	0.08	-9 (-17, 1)	-8 (-16, 2)	-0.02 (0.01)	0.10
Percentage morphologically normal spermatozoa	152	9 (5–13)	7 (4–12)	9 (4–13)	-0.05	0.54	-24 (-45, 6)	-19 (-42, 13)	-0.05 (0.03)	0.13
Mean testicular volume (mL)	168	15 (12–20)	15 (11–20)	15 (11–19)	-0.06	0.41	1 (-12, 16)	-6 (-18, 8)	-0.01 (0.01)	0.62
Testosterone (nmol/L)	169	22 (18–25)	21 (17–24)	21 (18–26)	-0.03	0.70	-2 (-13, 10)	1 (-10, 12)	0.00 (0.01)	0.70
Estradiol (nmol/L)	169	0.09 (0.08–0.11)	0.09 (0.08–0.12)	0.10 (0.08–0.12)	0.11	0.15	1 (-11, 15)	7 (-6, 21)	0.02 (0.01)	0.17
LH (IU/L)	169	4.2 (3.1–5.7)	4.2 (3.1–5.2)	4.7 (3.8–5.7)	0.12	0.11	6 (-11, 27)	24 (4, 48)	0.04 (0.02)	0.03
FSH (IU/L)	169	2.6 (1.8–3.8)	3.1 (2.4–4.2))	3.3 (2.4–4.8)	0.17	0.03	15 (-8, 44)	31(5, 64)	0.06 (0.02)	0.01
Inhibin B (pg/mL)	169	224 (172–258)	213 (153–278)	223 (169–262)	-0.02	0.82	2 (-14, 21)	0 (-15, 18)	-0.02 (0.02)	0.19
SHBG (nmol/L)	169	26 (22–34)	26 (20–33)	26 (22–33)	-0.03	0.72	-4 (-16, 10)	-3 (-15, 12)	-0.01 (0.01)	0.44
FAI	169	78 (67–94)	82 (66–101)	81 (67–99)	0.02	0.85	2 (-11, 17)	3 (-10, 18)	0.01 (0.01)	0.66

[a]Spearman's rank correlation coefficient and p-value for PFOA (continuous) and untransformed outcomes.

[b]Adjustment: All multivariable regression results were adjusted for history of reproductive tract disease, the son's body mass index, son's smoking status, maternal smoking during pregnancy, and socioeconomic status. Sperm concentration, total sperm count, progressive spermatozoa, semen volume, and testicular volume were adjusted for abstinence time; sperm concentration was also adjusted for spillage during semen sample collection; progressive spermatozoa was also adjusted for time from ejaculation to semen analysis; reproductive hormones were also adjusted for time of day of blood sampling.

[c] Beta coefficient for PFOA modelled as a continuous variable in a multivariable linear regression model of ln-transformed outcomes with adjustment for covariates as indicated above, and p-value as a test of linear trend.

Note: p=percentile. Number of participants in each regression analysis depended on the outcome variable and missing data in the covariates

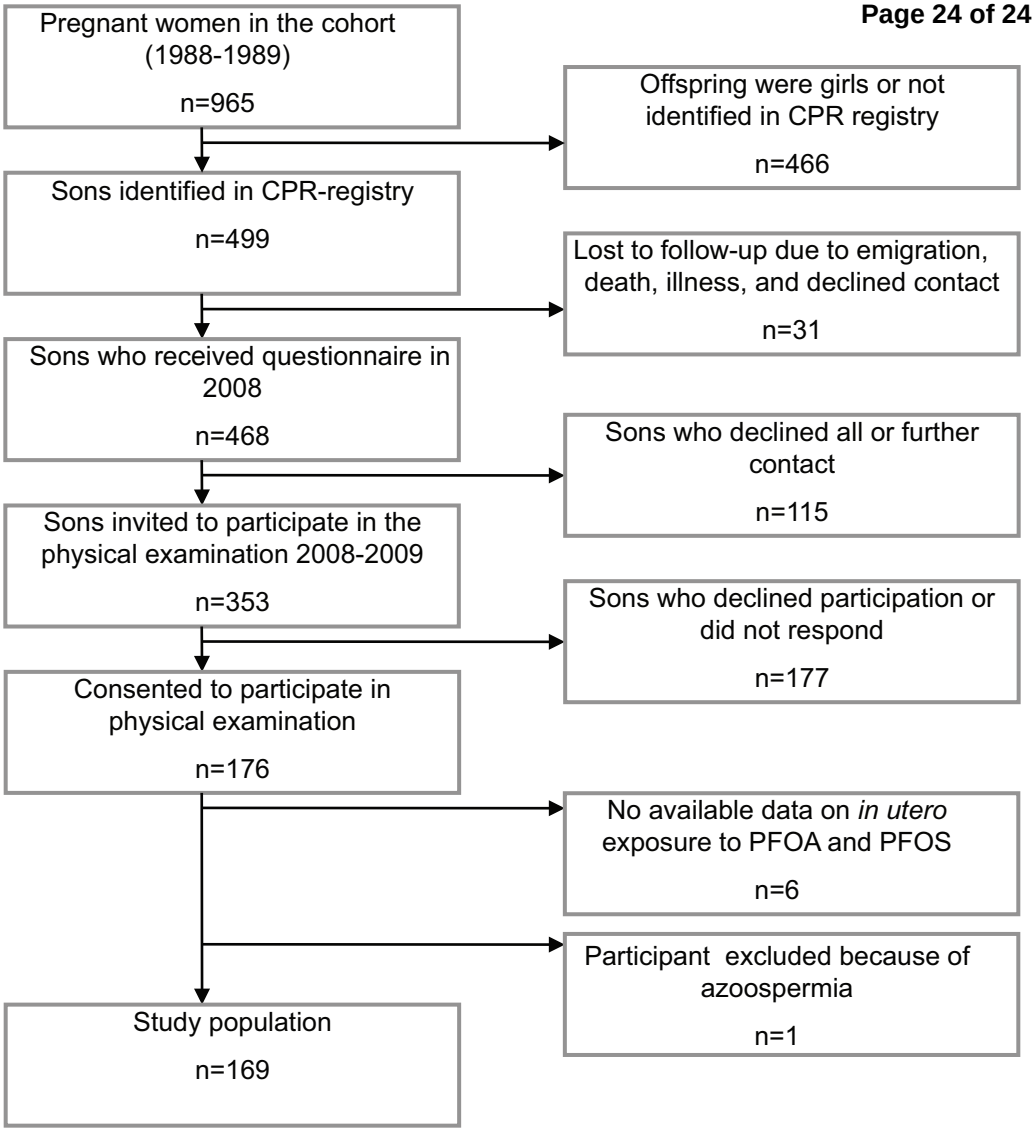
Table 3: Semen-, testicular size-, and reproductive hormone characteristics for 169 young Danish men stratified by tertiles of maternal pregnancy week 30 serum PFOS concentrations.

Parameter	n	Low PFOS: Median (p25–p75)	Medium PFOS: Median (p25–p75)	High PFOS: Median (p25–p75)	Spearman corr. coeff[a].	Trend p-value[a]	Medium PFOS: % difference from low (95% CI) [b]	High PFOS: % difference from low (95% CI) [b]	Adjusted β-coeff.[c] (SE.)	Adjusted trend p-value[c]
Sperm concentration (mio/mL)	168	35 (19–58)	32 (12–62)	37 (17–94)	0.00	0.99	-24 (-52, 21)	-1 (-38, 59)	-0.01 (0.01)	0.37
Total sperm count (mio)	123	103 (55–176)	77 (42–204)	124 (50–244)	0.01	0.87	-36 (-64, 12)	-23 (-56, 38)	-0.02 (0.01)	0.12
Semen volume (mL)	123	3 (2–4)	3 (2–4)	3 (2–4)	0.05	0.56	-8 (-26, 13)	-5 (-24, 18)	0.00 (0.01)	0.58
Percentage progressive spermatozoa	167	66 (57–74)	67 (54–76)	63 (52–70)	-0.13	0.10	0 (-9, 10)	-7 (-16, 2)	0.00 (0.00)	0.17
Percentage morphologically normal spermatozoa	152	9 (4–13)	8 (4–14)	9 (4–12)	-0.05	0.57	-4 (-31, 34)	-14 (-39, 20)	-0.01 (0.01)	0.31
Mean testicular volume (mL)	168	15 (12–20)	14 (11–20)	15 (12–20)	-0.03	0.69	-9 (21, 4)	-4 (-17, 11)	0.00 (0.00)	0.52
Testosterone (nmol/L)	169	22 (19–26)	20 (16–24)	21 (18–25)	-0.05	0.50	-10 (-19, 1)	-5 (-15, 6)	0.00 (0.00)	0.87
Estradiol (nmol/L)	169	0.10 (0.08–0.12)	0.09 (0.08–0.11)	0.1 (0.08–0.12)	0.06	0.45	-7 (-19, 5)	1 (-11, 16)	0.00 (0.00)	0.27
LH (IU/L)	169	4 (4–6)	4 (3–5)	5 (4–6)	0.00	0.93	-7 (-22, 12)	-2 (-18, 18)	0.00 (0.00)	0.95
FSH (IU/L)	169	3 (2– 4)	3 (2–4)	3 (2–5)	0.12	0.13	3 (-18, 29)	20 (-5, 51)	0.01 (0.01)	0.06
Inhibin B (pg/mL)	169	225 (163–256)	221 (148–274)	214 (171–266)	-0.01	0.91	-6 (-20, 11)	0 (-16, 19)	0.00 (0.00)	0.72
SHBG (nmol/L)	169	26 (22–35)	25 (20–30)	29 (22–36)	0.01	0.89	-10 (-22, 2)	5 (-8, 20)	0.00 (0.00)	0.66
FAI	169	81 (67–103)	84 (68–98)	77 (66–94)	-0.03	0.68	1 (-12, 16)	-10 (-21, 4)	0.00 (0.00)	0.57

[a]Spearman's rank correlation coefficient and p-value for PFOS (continuous) and untransformed outcomes. [b]Adjustment: All multivariable regression results were adjusted for history of reproductive tract disease, the son's body mass index, son's smoking status, maternal smoking during pregnancy, and socioeconomic status. Sperm concentration, total sperm count, progressive spermatozoa, semen volume, and testicular volume were adjusted for abstinence time; sperm concentration was also adjusted for spillage during semen sample collection; progressive spermatozoa was also adjusted for time from ejaculation to semen analysis; and the reproductive hormones were also adjusted for time of day of blood sampling. [c] Beta coefficient for PFOS modelled as a continuous variable in a multivariable linear regression model of ln-transformed outcomes with adjustment for covariates as indicated above, and p-value as a test of linear trend. Note: P=percentile. Number of participants in each regression analysis depended on the outcome variable and missing data in the covariates

Figure legend

Figure 1
Flow chart of recruitment to the physical examination of the follow-up study


Pregnant women in the cohort (1988-1989)
n=965
Offspring were girls or not identified in CPR registry
n=466
Sons identified in CPR-registry
n=499
Lost to follow-up due to emigration, death, illness, and declined contact
n=31
Sons who received questionnaire in 2008
n=468
Sons who declined all or further contact
n=115
Sons invited to participate in the physical examination 2008-2009
n=353
Sons who declined participation or did not respond
n=177
Consented to participate in physical examination
n=176
No available data on *in utero* exposure to PFOA and PFOS
n=6
Participant excluded because of azoospermia
n=1
Study population
n=169